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Dual-Color Detection of DNA Sequence Variants by Ligase-Mediated Analysis

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Genetic screening for sequence variants associated with disease is assuming increasing importance in clinical medicine as well as in research. We describe an efficient method for such analyses, comprising a combination of practical features: (1) Amplified DNA samples are analyzed for their ability to serve as templates in standardized allele-specific ligation reactions between oligonucleotide probes; (2) Two allele-specific probes, differentially labeled with either of two lanthanide labels, compete for ligation to a third oligonucleotide (the signal from the two labeled probes can thus be directly compared in a sensitive time-resolved fluorescence detection reaction); and (3) Large sets of analyses are processed in parallel using a 96-pin capture manifold, serving to reduce pipetting steps and the risk of contamination. We present here the basis of the technique and its application to the screening for two common mutations causing cystic fibrosis and α_1-antiytrypsin deficiency. © 1994 Academic Press, Inc.

INTRODUCTION

The presence of specific nucleotide sequence variants is frequently of predictive value in clinical medicine: many genetic diseases are caused by a limited number of different mutations in given human populations. In malignancies, analysis of specific mutations may be helpful in identifying and localizing tumor cells in a patient. In infectious diseases such as AIDS, specific sets of mutations are consistently observed in drug-resistant variants of the infectious agents (Larder and Kemp, 1989). Identification of gene variants is also important in tissue transplantation and in forensic medicine. A large number of methods that permit distinction among known DNA sequence variants have been described (for a recent review, see Cotton, 1993). Such methods include sequence-specific oligonucleotide hybridization (Wallace *et al.*, 1979), differential priming by matched or mismatched oligonucleotides during PCR (Newton *et al.*, 1989), selective incorporation of an allele-specific nucleotide downstream of a primer in minisequencing (Syvänen *et al.*, 1990), and target-dependent probe-ligation in the oligonucleotide ligation assay (OLA) (Landegren *et al.*, 1988). All of the above techniques can be used to distinguish nucleotide sequence variants, but the techniques differ, however, with respect to the convenience of the assay formats. Methods suitable for routine mutation screening must work under standardized conditions and should permit a high throughput. Ideally, the detection reaction should offer some form of internal control. It is also desirable that analytic procedures based on amplification minimize the risk of contamination and allow efficient processing of large sets of samples. Here, we present a robust analytic strategy that satisfies these requirements. The strategy is based on target-dependent oligonucleotide ligation performed using a 96-pin capture device and analyzed by dual-color time-resolved fluorometry.

MATERIALS AND METHODS

DNA samples. Samples from individuals diagnosed as being normal, carriers, or suffering from cystic fibrosis (Riordan *et al.*, 1989) were kindly provided by Dr. Niklas Dahl. DNA samples from individuals that are normal, carriers, or homozygous with respect to the Z mutation of the α_1-antitrypsin gene (Laurell and Eriksson, 1963) were generously made available by Dr. Fielding Heitmançik.

Oligonucleotides. Allele-specific oligonucleotides were labeled at their 5′ ends with a series of chelates of europium or terbium ions by incorporating amino-modified nucleotides during oligonucleotide synthesis and reacting them with an isothiocyanate-derivatized chelating agent (Dahlén *et al.*, 1991). Excess chelates were removed by gel chromatography on a Superose 12 column (Pharmacia, Uppsala). Alternatively, chelating agents were introduced as phosphoramidites during oligonucleotide synthesis (M. K., manuscript in preparation). Biotin groups were incorporated at the 3′ ends of oligonucleotides, modified at the 5′ termini with phosphate. The oligonucleotides were purified by reverse-phase FPLC.

Oligonucleotides used to amplify the Δ508 CF mutation were 5′-GTTTTCCTGGATTATGCCTGGCAC-3′ and 5′-GTTGGCATGCTTTGATGACGCTTC-3′; and those for the α_1-antitrypsin mutation were 5′-TCAGCCTTACAACGTGTCTCTGCTT-3′ and 5′-GTATGGCCCTCTAAAAACATGGCCCC-3′. Three additional oligonucleotides were designed to hybridize to each amplification product. Two of the oligonucleotides were 5′-labeled with 10 europium chelates or 15 terbium chelates. These oligonucleotides, specific for the normal or the mutant variant of the gene, respectively, were compared for their ability to ligate to a third oligonucleotide. The third oligonu-

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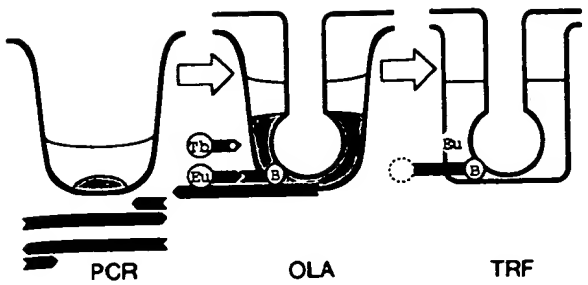

FIG. 1. Schematic description of the mutation detection assay. The sequence to be analyzed for the presence of specific mutations is amplified by PCR. After this, a set of three oligonucleotides is introduced, along with a ligase, and the oligonucleotides are collected on a 96-pin capture manifold. Two of the oligonucleotides have different 3′ ends (indicated by an open circle in one of the probes). These two oligonucleotides are specific for the normal or a mutant variant of a gene and they are differentially labeled with chelated europium or terbium ions, respectively. After the ligation reaction, the supports are washed, and collected oligonucleotides are transferred to separate microtiter wells in which europium and terbium ions are released. The fluorescence from chelated europium ions is then determined for each well, followed by a measurement of the terbium fluorescence.

cleotide was designed to hybridize immediately downstream of the first two and was equipped with a 5′ phosphate and a biotin group at the 3′ position. For the CF gene, the two lanthanide-labeled oligonucleotides were Eu_{10} 5′-ATTAAAGAAAATATCATCTT-3′ and Tb_{15} 5′-ACCATTAAAGAAAATATCAT-3′, and the third oligonucleotide was P5′-TGGTGTTTCCTATGATGAAT-3′B. The corresponding oligonucleotides used to analyze the α_1-antitrypsin muta tion were Eu_{10} 5′-GGCTGTGCTGACCATCGACG-3′, Tb_{15} 5′-GGCTGTGCTGACCATCGACA-3′, and P5′-AGAAAGGGACTGAAGCTGCT-3′B.

PCR. Amplification reactions were performed in 100 μl, and 4-μl aliquots were distributed for ligation analysis. For screening purposes, 2 μl of DNA samples at 2 ng/μl was mixed with 2 μl of the two oligonucleotides used for amplification at 1 μ*M* each and 0.1 U/μl of *Taq* polymerase. Both the DNA samples and the amplification reagents were added in a buffer of 50 m*M* KCl, 50 m*M* Tris–HCl, pH 8.3, and 1.5 m*M* $MgCl_2$ to avoid minor fluctuations in buffer composition due to inaccuracies in the small volumes added. The amplification reactions were performed in wells of a polyvinyl microtiter plate (Falcon, Oxnard, CA), overlayered with mineral oil, for 30 temperature cycles at 94°C, 55°C, and 72°C of 60 s each.

Oligonucleotide ligation assay. After amplification, individual 4-μl amplification reactions were diluted to 10 μl with distilled water and heated to 94°C for 3 min in the thermal cycler used for amplification. After the temperature had been rapidly brought back to 37°C, a 10-μl ligation mix, including three oligonucleotides, was added. One oligonucleotide, specific for the normal allele, was labeled with europium ions, and another, a mutant-specific oligonucleotide, was terbium-labeled. The third oligonucleotide was designed to hybridize downstream of either of the allele-specific oligonucleotides, and it had a biotin group at the 3′ end. This oligonucleotide also had a 5′ phosphate group, which is required in the ligation reaction. Six hundred femtomoles of each of the three oligonucleotides was added per reaction in 10 μl of 10 m*M* Tris–HCl, pH 7.5, 400 m*M* NaCl, 50 m*M* KCl, 10 m*M* $MgCl_2$, 1 m*M* ATP, and 40 mU T4 DNA ligase (Pharmacia). After a 30-min incubation at 37°C, 20 μl of binding buffer (1 *M* NaCl, 100 m*M* Tris–HCl, pH 7.5, and 0.1% Triton X-100) was added. The ligation products were then captured on solid supports as described below.

Solid support. Biotinylated ligation products were collected on an avidin-coated 96-pin capture manifold. The supports were prepared as previously described (Parik *et al.*, 1993). Briefly, avidin was coupled to NHS-activated Sepharose particles, and the methanol-dried particles were suspended in an organic solvent, triethylamine. A commercially available polystyrene support, with a set of 96 prongs designed to fit into individual microtiter wells (Falcon), was briefly immersed in the particle suspension. In this manner, the surface of the prongs was greatly expanded through the attachment of the porous Sepharose particles. The supports were then washed and blocked in 0.5% dry milk in 10 m*M* Tris–HCl, pH 7.5, 50 m*M* KCl. Shortly before use, supports were washed and then incubated in ligation reactions in microtiter wells for 30 min on a shaking platform.

Time-resolved fluorometry. After two washes of the supports in binding buffer for 3 min each, followed by 10 min in 1 *M* NaCl, 0.1 *M* NaOH, and 0.1% Triton X-100, and then two more washes in binding buffer, the supports were transferred to a flat-bottom polystyrene microtiter plate (Nunc, Denmark) containing 180 μl of a fluorescence enhancement solution (0.1 *M* acetate-phthalate, pH 3.2, 15 μ*M* 2-naphtoyl trifluoroacetone, 50 m*M* tri-*N*-octylphosphine oxide, and 0.1% Triton X-100; Wallac, Finland). At the low pH of this solution, lanthanide ions are released from oligonucleotides bound to the supports. The supports were removed after 15 min and the fluorescence from the europium chelates that form in the enhancement solution was quantitated in a Delfia Plate Reader Research Fluorometer (Wallac). Next, 20 μl of terbium enhancement was added to the wells and, after a further 10-min incubation, the terbium-specific fluorescence was recorded, as recommended by the manufacturer. This enhancement solution was prepared by a modification of a previously described procedure (Hale, 1990) and consisted of 100 μ*M* 4-(2,4,6-trimethoxyphenyl)-pyridine-2,6-dicarboxylic acid and 1% cetyltrimethylammonium bromide in 1.1 *M* $NaHCO_3$. The fluorescence from europium ions results in an approximately 20-fold stronger reading but, due to differences in background, the two ions exhibit similar detection sensitivity.

RESULTS

Strategy for the Analysis of Gene Sequence Variants

The assay for distinction among sequence variants in DNA samples, amplified by PCR, includes the following steps (Fig. 1): After the amplification reaction, samples are diluted and denatured, and for each reaction, a set of three oligonucleotides is added, along with a ligase. Two of the oligonucleotides are of similar sequence but differ at their 3′ ends. One of these oligonucleotides base-pairs correctly with a normal gene sequence. At the 5′ end of this oligonucleotide, a series of europium chelates have been added. The other oligonucleotide, of similar sequence but specific for a frequent mutation in the gene in question, is 5′-labeled with terbium chelates. Depending on whether the sample was derived from an individual homozygous for either sequence variant or heterozygous, one or both of the two chelate-modified oligonucleotides can be ligated to the third oligonucleotide probe. This probe hybridizes immediately downstream of the other two and it has a 5′ phosphate group and a biotin at the 3′ position. After ligation, biotinylated molecules are trapped on a multipronged support projecting into individual microtiter wells.

Detection of Variable Amounts of Amplification Products

It is important for an amplification-based gene analytic procedure to be relatively independent of the amount of product generated in an amplification reaction, since this may vary between reactions. By using dual-color detection, an internal control is obtained in each individual assay permitting direct comparison

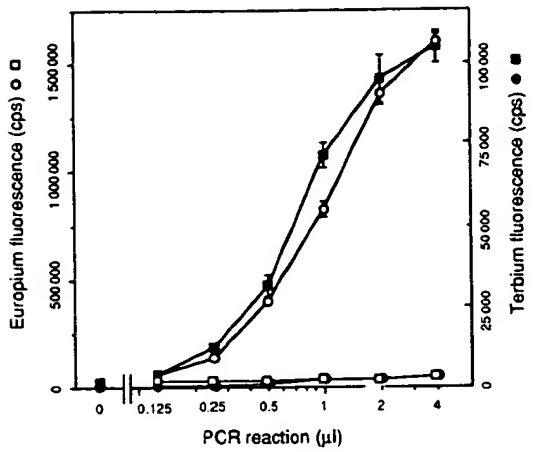

FIG. 2. Detection of variable amounts of PCR reaction products. Amplification reactions from individuals homozygous for the normal gene sequence (circles) or for the ΔF508 variant of the CFTR gene (squares) were serially diluted and analyzed in a dual-color ligation reaction. The average europium- and terbium-specific fluorescence detected in triplicate assays (open and filled symbols, respectively) is given with the standard error as a function of the volume of PCR reaction investigated.

among the signals for the two alleles. We investigated the ability of the assay to identify the common ΔF508 mutation of the cystic fibrosis transmembrane regulator (CFTR) gene in variable polymerase chain reaction (PCR) volumes. In Fig. 2 the europium and terbium fluorescence signals from a set of ligation reactions are shown as a function of the amount of PCR product analyzed. The experiment demonstrates that ligation reactions performed with an amplified DNA sample from an individual homozygous for the normal CFTR gene result in prominent europium signals but low terbium readings. This indicates that in the presence of the normal sequence, europium-labeled oligonucleotides are ligated to the downstream oligonucleotide. Conversely, ligation reactions performed with a sample derived from an individual who is homozygous for the ΔF508 mutation result in terbium signals. As little as 0.25 µl of the amplification reaction is sufficient to unambiguously distinguish these genotypes. This corresponds to approximately 40 nmol of PCR product, based on the estimation of ethidium bromide-stained PCR products. In the subsequent experiments, we have used 4-µl PCR samples per ligation assay.

Ligase Dependence of the Detection Reactions

An assay used to identify sequence variants should ideally serve to distinguish any allelic sequences under a wide range of reaction conditions. We examined how robust the ligation assay is to a range of ligase concentration in reactions established in the presence of amplified DNA samples from individuals normal or homozygous with respect to two common mutations. Figure 3 illustrates the ligase-mediated identification of two mutations causing cystic fibrosis and α_1-antitrypsin deficiency, respectively. Over a more than 10,000-fold range of ligase concentrations, the assay accurately distinguishes sequence variants in both genes. Detection of the mutation causing α_1-antitrypsin deficiency requires

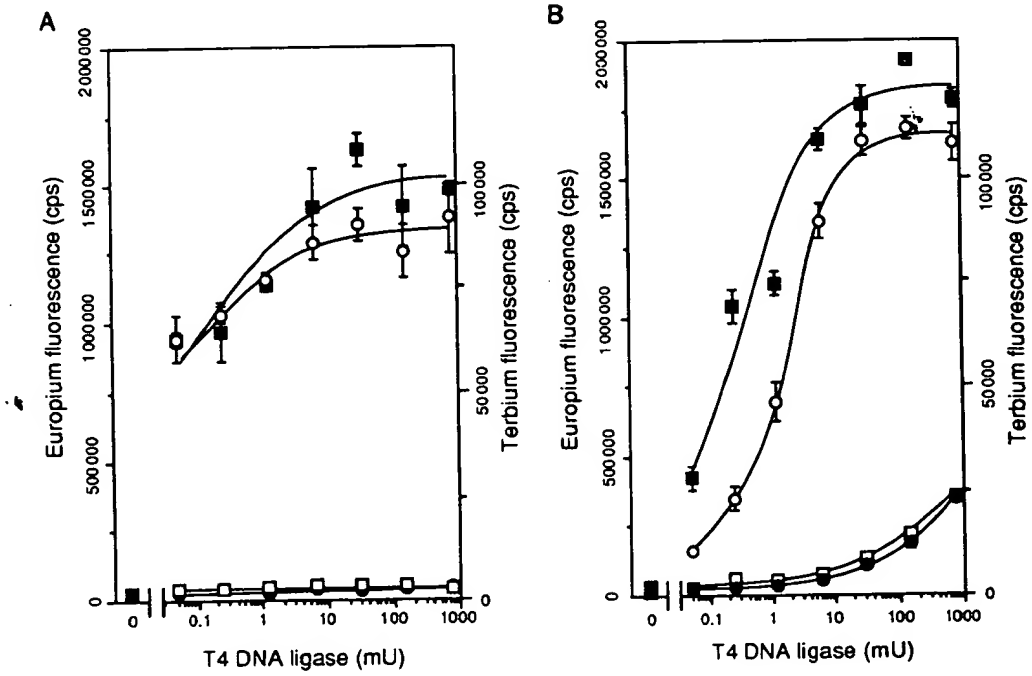

FIG. 3. Dependence of the allele-specific ligation on the amount of T4 DNA ligase added to the ligation reactions. Frequent mutations in the two common genetic diseases (A) cystic fibrosis and (B) α_1-antitrypsin deficiency were studied. Samples amplified from individuals homozygous for the mutation (open circles) or for the normal sequence (filled circles) were compared. Europium readings, representing reagents specific for the normal sequence, are indicated by open symbols. Terbium readings, representing reagents specific for the mutant sequence, are shown as filled symbols. The results are given as the mean and standard deviation of triplicate samples.

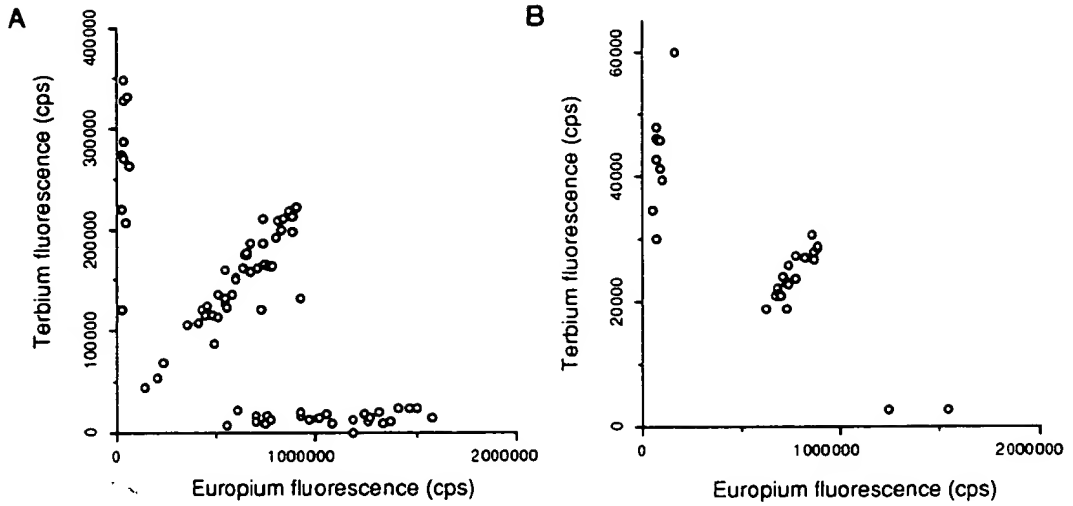

FIG. 4. Screening for common mutations causing genetic disease. (A) Seventy-five DNA samples obtained from cystic fibrosis patients and their immediate relatives were screened for the ΔF508 mutation in the cystic fibrosis transmembrane conductance regulator gene. (B) A group of 27 patients with known abnormalities of α_1-antitrypsin were analyzed for the presence of the Z mutation, using the ligase-based assay. The europium and terbium fluorescence readings from dual-color assays from individual DNA samples, performed in triplicate, are indicated by open circles.

identification of a G:T basepair. This probe–target mismatch is adequately distinguished from the perfect match, despite that this mismatch is notoriously difficult to distinguish from a regular Watson Crick basepair in many different assays (see, for example, Kwok *et al.*, 1990). It is of interest to note that at very high ligase concentrations, the CF mutation, which involves the loss of three contiguous nucleotide positions, is still completely discriminated, whereas for the α_1-antitrypsin gene some probes mismatched at a single position become joined. Nonetheless, a wide range of ligase concentrations can be used to distinguish the sequence variants.

Screening for Mutations Causing CF and α_1-antitrypsin Deficiency

On the basis of results from experiments to optimize reaction conditions in the ligation assay, we investigated in a practical application DNA samples from patients and their relatives for mutations causing the same two relatively frequent monogenic diseases. The europium and terbium fluorescence readings from individual reactions are shown in Fig. 4. Three clearly resolved clusters of data points are noted for both loci studied. Samples derived from individuals that are homozygous for the normal sequence variants predominantly result in europium signals and affected individuals homozygous for the mutant sequences primarily result in terbium signals. Samples from heterozygous carriers of the investigated mutations fall along a diagonal with both terbium and europium signals. Any samples for which the combined signals do not exceed a critical threshold are identified as reactions in which the amplification reaction may have failed. Genotypes of positive reactions can then be calculated by comparing the ratios between europium and terbium signals in individual samples.

DISCUSSION

The present mutation detection assay is a robust and practical method for analyzing large sets of DNA samples for defined mutations. The ligation reaction used to distinguish sequence variants affords an efficient distinction under standardized reaction conditions (Landegren *et al.*, 1988). The method has previously been shown to be suitable for automated genetic assays (Nickerson *et al.*, 1990, 1992). Ligase-based analyses also offer other advantages, such as the high specificity of detection resulting from the requirement that two oligonucleotides hybridize in juxtaposition on a target strand. This specificity is adequate to uniquely identify single-copy sequences in total human genomic DNA, given sufficiently sensitive detection reactions (Landegren *et al.*, 1988; Alves and Carr, 1988; Barany 1991). Moreover, the covalent bond generated between the two probe halves is stable to extensive washes, serving to reduce nonspecific binding of probe molecules.

Time-resolved fluorescence detection presents several attractive properties in the visualization step of gene analytic reactions: for enhanced sensitivity of detection, series of lanthanide labels are introduced in a simple conjugation reaction to amino-modified oligonucleotides. Alternatively, this type of label can be introduced during standard oligonucleotide synthesis (M.K. *et al.*, manuscript in preparation). The chelates of europium and terbium are quite stable to storage and to the reaction conditions. Specifically, minimal exchange of europium and terbium ions is observed between the differentially labeled oligonucleotides under the conditions used (M.K. *et al.*, manuscript in preparation). The dual-color design offers convenient internal controls in the detection reactions and reduces the requirement for replicate analyses. Using the same chemistry and instrumentation, at least three different markers can be investigated

simultaneously by also using samarium-labeled reagents. Dual-labeling with lanthanide chelates has been previously described in the context of sequence-specific oligonucleotide hybridization analysis (Iitiä *et al.*, 1992).

The 96-pin capture manifold used in this study is also of value in routine diagnostic assays. It permits efficient processing of large sets of samples through sequential reaction steps, and it reduces the risk of contamination and sample mix-ups. Through the expanded surface available on the supports, a greatly increased binding capacity that permits binding several tens of picomoles of biotinylated oligonucleotides results (Parik *et al.*, 1993). The manifold support, perhaps used in conjunction with dual-color rare earth metal labels, will be useful in many different genetic analyses. The same assay format presented here is of value to detect specific sequences, and the dual-color design is of value in quantitative investigations of gene expression. Using preali-quoted, stable reagents, a very simple, standard analysis format requiring few or no pipetting steps would result (Ortlepp and McKay, 1989). The solid phases used here have also proven valuable for template preparation, processing, and loading of reactions in DNA sequencing (Lagerkvist *et al.*, 1994). In conclusion, the present assay combines features that should prove to be of general value in DNA diagnostic investigations.

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